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Syntheses of bifunctional molecules containing [12]aneN$_3$ and carbazol moieties as effective DNA condensation agents

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Bifunctional molecules containing macrocyclic polyamine [12]aneN$_3$ and carbazol units, **1**–**4,** have been efficiently synthesized and fully characterized. Through gel electrophoresis, atomic force microscopy, and dynamic light scattering experiments, compounds **3** and **4b** bearing both [12]aneN$_3$ and carbazol moieties showed effective DNA condensation ability at the concentration of 80 μM. Investigations from EB displacement fluorescence spectra, viscosity titration, and ionic strength effects revealed that the effective DNA condensation comes from the appropriate combination of carbazol and [12]aneN$_3$ units in the bifunctional molecules, and the DNA condensation process is reversible. The incorporation of triazole units in the molecules clearly reduced the cytotoxicity.

DNA condensation, macrocyclic polyamines, carbazoles, triazole, gel electrophoresis, AFM

1 Introduction

The condensation of DNA, a process in which the extended DNA chains are collapsed into tightly packed state, has received considerable attention due to its biological importance in DNA transcription, replication, and gene therapy [1–4]. Various compounds including cationic lipids, polymers, dendrimers, peptides, chitosan, and metal complexes have been synthesized as DNA condensing agents and used as non-viral gene vectors in *in vitro* experiments and clinic trials for the last two decades [5–12]. Compared to viral gene vectors, these synthetic agents have shown the advantages including biocompatibility, non-immunogenicity, convenience for modification, and potential for large-scale production. Tremendous progress has been achieved in the design and synthesis of non-viral vectors, some common design principles and structure-property trends have emerged. For example, almost all synthetic gene vectors contain amine moieties which can be protonated to afford electrostatic binding with DNA. The structure features and charge density of the condensers have remarkable effects on the kinetic process of DNA condensation and the size of the nanoparticles formed [5, 13–17]. However, there are still several barriers to be overcome before the synthetic non-viral vectors can be put into practical application. These include the lack of reproducible and scalable formulation, low stability in biological fluids, proneness to aggregation, long term storage, DNA size-dependent delivery, and properly reproducible and efficient transfection. Thus it is desirable to explore new and novel DNA condensing agents as potential non-viral gene vectors.

The interactions between DNA and small molecules are categorized into three main modes: electrostatic binding, groove-binding, and intercalative-binding. For most organic condensing agents, the presence of positively charged moieties such as amine or guanidine groups is prerequisite. Recently, it was found that the intercalative binding could be the major contribution in the condensation process for the metal complexes condensing agents [18, 19], which was

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also true for the early discovered dye-type condensers [20, 21]. Based on the above consideration, we are currently working on the design and synthesis of bifunctional molecules as effective DNA condensing agents [22, 23]. Herein, we report the synthesis of a series of bifunctional molecules bearing macrocyclic polyamine [12]aneN_3 and carbazole moieties (Figure 1) and their application in the condensation of DNA. Macrocyclic polyamines have been widely applied in the design and synthesis of artificial nucleases and non-viral gene vectors [12, 24–26], serving as electrostatic binding unit. Carbazole derivatives are often used as the intercalative molecules with DNA and for photo-physical studies [27, 28], with a large planar aromatic ring promoting the condensation process.

2 Experimental

2.1 Materials and methods

All solvents and reagents were of analytical grade and were used as received. Ultrapure milli-Q water (18.25 MΩ) was used in all DNA condensation assays. DNA was purchased from Solarbio company. The concentrations of CT-DNA were determined by UV spectroscopy at 260 nm, taking 6600 M^{-1} cm^{-1} as the molar absorption coefficient. ^{1}H and ^{13}C NMR spectra were obtained on an Advance Bruker 400 MHz spectrometer at 25 °C. Chemical shifts (in ppm) were reported relative to internal tetramethylsilane (TMS) or residual solvent peaks. IR spectra were recorded on a Nicolet 380 spectrometer in the range of 4000–400 cm^{-1}, samples were prepared as KBr and NaCl pellets. ESI-MS and HRMS spectra were recorded on Quattro Mocro and Bruker Daltonics Bio TOF mass spectrometer, respectively. AFM images were obtained with a Veeco NanoScope IIIa atomic force microscope. Hydrodynamic diameter distribution was tested using a DynaPro Nanostar dynamic laser light scattering apparatus. Fluorescence spectra were recorded on a Varian Cary Eclipse Spectrometer. UV-Vis spectra were measured on a Varian Cary 300 UV-Vis spectrophotometer. The viscosity was measured using a Ubblehode viscometer.

2.2 Synthesis

Compounds **4a**, **5**, **7** and **9** were prepared by following literature procedures [22, 25, 29–31].

*1-(1-Ethyl-1,2,3-triazol-4-ylmethyl)-1,5,9-triazacylododecane (**1**)*

To a solution of ethyl bromide (0.83 g, 7.6 mmol) in DMF (6 mL) was added sodium azide (1.39 g, 21.3 mmol). The suspension was stirred at 90 °C for 24 h, the reaction was cooled to room temperature, then compound **7** (0.80 g, 2.0 mmol), $CuSO_4$ (64 mg, 0.40 mmol) and sodium ascorbate (160 mg, 0.80 mmol) were added. The resulting mixture was stirred at room temperature under argon for 12 h. Then the mixture was poured into water and filtered. The solid was washed with water and dried in vacuo, after which the solid was added into HCl solution (Prepared by slowly adding 0.5 mL of acetyl chloride into 8 mL of methanol and then stirring for 5 min at 0 °C) and stirred for 1 h. Then the solvents were removed in vacuo and the resulting solid was dried to give **1** as its hydrochloric salt (0.54 g, 70%). ^{1}H NMR (400 MHz, D_2O) δ 8.05 (s, 1H), 4.34 (q, J = 7.4 Hz, 2H), 4.23 (s, 2H), 3.22 (dd, J = 12.8, 6.3 Hz, 8H), 3.13 (s, 4H), 2.19–2.11 (m, 2H), 2.06 (s, 4H), 1.37 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, D_2O) δ 137.92, 126.22, 48.41, 47.93, 46.01, 42.86, 41.65, 19.91, 18.68, 14.48; IR (KBr): ν 751 (w), 855 (w), 906 (w), 1002 (w), 1055 (m), 1152 (w), 1218 (w), 1364 (w), 1458 (m), 1587 (m), 2757 (s), 2954 (s), 3422 cm^{-1} (s); HRMS (ES+) calcd. for $C_{14}H_{29}N_6$ $(M+H)^+$: 281.2454, found 281.2452.

*N-(2-azidoethyl)carbazol (**6a**)*

To a solution of **5a** (0.262 g, 0.956 mmol) in DMF (8 mL) was added sodium azide (0.620 g, 9.56 mmol). The suspension was stirred at 80 °C for 8 h. After being cooled down to room temperature, the mixture was poured into water (100 mL), stirred for 5 min and then filtered. The solid was dried in vacuo (0.210 g, 93%) and used without any further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, J = 7.8, 2H), 7.47 (m, 4H), 7.26 (t, J = 6.4, 2H), 4.48 (t, J = 6.2, 2H), 3.73 (t, J = 6.2, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.16, 125.93, 123.16, 120.54, 119.47, 108.39, 49.88, 42.36; MS (ES+): m/z = 259.5 $[M+Na]^+$.

*N-(3-azidopropyl)carbazol (**6b**)*

To a solution of **5b** (0.288 g, 1 mmol) in DMF (8 mL) was added sodium azide (0.650 g, 10 mmol). The suspension was stirred at 80 °C for 6 h. After being cooled to room temperature, a saturated aqueous NaCl (30 mL) was added and the solution was extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were washed with

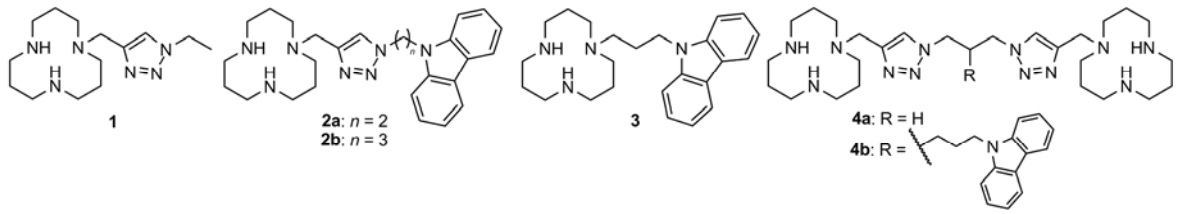

Figure 1 Structures of bifunctional molecules.

saturated aqueous NaCl (3 × 30 mL), dried over $NaSO_4$ and concentrated in vacuo. The crude product was purified by chromatography (eluting with PE: DCM, *v*/*v* = 5:1) to give **5b** as a colorless oil (0.239 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, *J* = 7.8, 2H), 7.48 (m, 2H), 7.44 (d, *J* = 8.0, 2H), 7.25 (d, *J* = 8.2, 2H), 4.42 (t, *J* = 6.5, 2H), 3.30 (t, *J* = 6.3, 2H), 2.14 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.28, 125.84, 122.92, 120.44, 119.10, 108.44, 48.62, 39.66, 28.18; MS (ES+): *m*/*z* = 253.2 $[M+H]^+$.

*N-{2-[4-(1,5,9-triazacylododec-1-ylmethyl)-1,2,3-triazol-1-yl]ethyl}carbazol (**2a**)*

To a solution of **7** (54 mg, 0.13 mmol) in H_2O/DMF (1/2, 3 mL) were added **6a** (31 mg, 0.13 mmol), $CuSO_4$ (3 mg, 0.02 mmol), and sodium ascorbate (8 mg, 0.04 mmol). The reaction was stirred at room temperature for 8 h. Then the mixture was poured into water and filtered. The solid was washed with water and dried in vacuo, after which the solid was added into HCl solution (Prepared by slowly adding 0.5 mL of acetyl chloride into 8 mL of methanol and then stirring for 5 min at 0 °C) and stirred for 1 h. Then the solvents were removed in vacuo and the resulting solid was dried to give **2a** as its hydrochloric salt (63 mg, 78%). ^{1}H NMR (400 MHz, D_2O) δ 7.92 (d, *J* = 7.7, 2H), 7.26 (t, *J* = 7.4, 2H), 7.10 (m, 4H), 4.78 (t, *J* = 5.1, 2H), 4.61 (t, *J* = 5.1, 2H), 3.46 (s, 2H), 3.05 (t, *J* = 5.5, 4H), 2.92 (t, *J* = 5.7, 4H), 2.06 (m, 6H), 1.62 (t, *J* = 5.5, 4H); ^{13}C NMR (101 MHz, D_2O) δ 139.45, 138.57, 126.46, 126.11, 122.12, 120.25, 119.53, 108.54, 48.98, 48.46, 44.57, 43.65, 42.12, 41.98, 19.51, 19.44; IR (KBr): ν 725 (m), 751 (s), 852 (w), 1046 (w), 1071 (w), 1123 (m), 1226 (m), 1287 (m), 1326 (m), 1351 (m), 1458 (s), 1485 (m), 1597 (w), 1626 (w), 2812 (m), 2928 (m), 3050 (w), 3432 cm^{-1} (w); HRMS (ES+) calcd. for $C_{26}H_{36}N_7$ $[M+H]^+$: 446.3032, found 446.3016.

*N-{3-[4-(1,5,9-triazacylododec-1-ylmethyl)-1,2,3-triazol-1-yl]propyl}carbazol (**2b**)*

To a solution of **7** (64 mg, 0.16 mmol) in H_2O/DMF (1/2, 3 mL) were added **6b** (40 mg, 0.16 mmol), $CuSO_4$ (3 mg, 0.02 mmol), and sodium ascorbate (8 mg, 0.04 mmol). The reaction was stirred at room temperature for 8 h. Then the mixture was poured into water and filtered. The solid was washed with water and dried in vacuo, after which the solid was added into HCl solution (Prepared by slowly adding 0.5 mL of acetyl chloride into 8 mL of methanol and then stirring for 5 min at 0 °C) and stirred for 1 h. Then the solvents were removed in vacuo and the resulting solid was dried to give **2b** as its hydrochloric salt (98 mg, 81%). ^{1}H NMR (400 MHz, D_2O) δ 7.61 (d, *J* = 7.3, 2H), 7.15 (t, *J* = 7.5, 2H), 7.03 (d, *J* = 7.7, 2H), 6.91 (t, *J* = 7.2, 2H), 6.83 (s, 1H), 3.851–4.02 (br, 4H), 3.45 (s, 2H), 3.20–2.90 (br, 8H), 2.40–2.70 (br, 4H), 1.90–2.20 (br, 4H), 1.70–1.85 (br, 4H); ^{13}C NMR (101 MHz, D_2O) δ 139.61, 137.11, 125.99, 125.19, 122.00, 119.97, 119.13, 109.02, 48.68, 46.53, 42.91, 41.60, 40.02, 27.34, 19.74, 18.89; IR (KBr): ν 619 (w), 725 (m), 752 (s), 1002 (w), 1023 (w), 1046 (w), 1130 (m), 1215 (m), 1327 (m), 1382 (w), 1454 (s), 1484 (m), 1596 (w), 1626 (w), 2809 (m), 2926 (m), 3050 (w), 3431 cm^{-1} (w); HRMS (ES+) calcd. for $C_{27}H_{38}N_7$ $[M+H]^+$: 460.3189, found 460.3199.

*1-[3-(9H-carbazol-9-yl)propyl]-1,5,9-triazacylododecane (**3**)*

To a solution of **5b** (1.44 g, 5 mmol) in toluene (15 mL) was added **9** (0.91 g, 5 mmol). The reaction was refluxed for 48 h. After being cooled down to room temperature, the resulting precipitates were filtered and taken into aqueous HCl solution (1 M, 20 mL). The mixture was refluxed for 6 h and allowed to cool to room temperature, after which time it was washed with $CHCl_3$ (3 × 20 mL) followed by the addition of concentrated aqueous NaOH solution, adjusting the pH to about 13. Then the mixture was extracted with $CHCl_3$ (3 × 20 mL) and the combined organic phase was dried over Na_2SO_4, filtered and concentrated to give **3** as a brown oil (1.80 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, *J* = 7.7, 2H), 7.46 (m, 4H), 7.22 (d, *J* = 6.7, 2H), 4.34 (t, *J* = 7.4, 2H), 2.72 (ddd, *J* = 27.3, 11.5, 5.3, 8H), 2.50 (t, *J* = 5.7, 6H), 2.04 (m, 2H), 1.62 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.41, 125.68, 122.97, 120.38, 118.86, 108.64, 52.89, 50.36, 49.33, 47.08, 41.61, 26.20, 25.63; IR (KBr): ν 728 (w), 756 (m), 1069 (w), 1154 (w), 1215 (w), 1327 (m), 1383 (w), 1454 (s), 1484 (m), 1594 (m), 1626 (w), 2747 (m), 2951 (m), 3421 cm^{-1} (m); HRMS (ES+) calcd. for $C_{24}H_{35}N_4$ $(M+H)^+$: 379.2862, found 379.2867.

*Diethyl 2-[3-(9H-carbazol-9-yl)]propylmalonate (**8a**)*

Metal sodium (0.23 g, 10 mmol) was cut into small pieces and added into absolute ethanol (30 mL) under argon. Once the reaction was complete, diethyl malonate (1.59 g, 10 mmol) was added, after which time a solution of compound **5b** (2.30 g, 8.0 mmol) in absolute ethanol (20 mL) was added dropwise. The reaction mixture was stirred at room temperature for 8 h. Then the solvent was removed in vacuo. The resulting oil was dissolved in water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo. The residual was purified by chromatography (eluting with ethyl acetate/petroleum ether, *v*/*v* = 1:10) to give **8a** as a white solid (2.55 g, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, *J* = 7.7 Hz, 2H), 7.49–7.42 (m, 2H), 7.39 (d, *J* = 8.2 Hz, 2H), 7.24–7.18 (m, 2H), 4.32 (t, *J* = 7.0 Hz, 2H), 4.14 (dtt, *J* = 10.8, 7.0, 3.7 Hz, 4H), 3.31 (t, *J* = 7.2 Hz, 1H), 2.05–1.86 (m, 4H), 1.20 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.07, 140.23, 125.70, 122.87, 120.37, 118.90, 108.51, 61.47, 51.58, 42.53, 26.60, 26.33, 13.99; IR (KBr): ν 610 (w), 724 (m), 760 (m), 867 (w), 939 (w), 1020 (m), 1053 (m), 1092 (m), 1149 (m), 1185 (m), 1254 (m), 1296 (m), 1326 (m), 1371 (m), 1452 (m), 1467 (m), 1479 (m), 1587 (w), 1620 (w), 1721 (s), 1739 (s), 2976

(m), 3060 cm^{-1} (w); MS (ESI+): *m/z* = 368.2 $[M+H]^+$.

*2-[3-(9H-Carbazol-9-yl)propyl]propane-1,3-diol (**8b**)*

Compound **8a** (3.73 g, 10.2 mmol) was dissolved in ethanol (20 mL) and $NaBH_4$ (2.30 g, 61.0 mmol) was added portionwise at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 12 h. When the reaction was complete, 10% aqueous HCl (5 mL) was added slowly and the mixture was stirred for a while. Then the solvents were removed in vacuo. The residue was dissolved in water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phase was dried over Na_2SO_4 and concentrated in vacuo. The crude product was then purified by chromatography (eluting with ethyl acetate/petroleum ether, *v*/*v* = 2:1) to give **8b** (1.83 g, 64%) as a colorless oil, which solidified after being stored in refrigerator. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J* = 7.7 Hz, 2H), 7.44–7.32 (m, 2H), 7.30 (d, *J* = 8.2 Hz, 2H), 7.15 (dd, *J* = 12.4, 4.7 Hz, 2H), 4.21 (t, *J* = 7.0 Hz, 2H), 3.62 (d, *J* = 8.9 Hz, 2H), 3.54–3.41 (m, 2H), 2.34 (s, 2H), 1.89–1.74 (m, 2H), 1.60 (s, 1H), 1.26 (dd, *J* = 15.5, 7.2 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.36, 125.69, 122.85, 120.40, 118.87, 108.60, 65.75, 43.10, 41.77, 26.65, 25.28; IR (KBr): ν 609 (w), 726 (m), 761 (s), 856 (w), 1020 (m), 1056 (m), 1098 (m), 1144 (m), 1179(w), 1257 (m), 1293 (m), 1330 (m), 1373 (m), 1453 (m), 1469 (m), 1477(m), 1589 (w), 1625 (w), 2958 (w), 3060 (vw), 3319 cm^{-1} (m); MS (ESI+): *m/z* = 284.8 $[M+H]^+$.

*2-[3-(9H-Carbazol-9-yl)propyl]-1,3-bistosyloxypropane (**8c**)*

To a solution of compound **8b** (0.94 g, 3.3 mmol) in THF (10 mL) was added 10% aqueous NaOH solution (15 mL). The mixture was stirred at 0 °C for half an hour. Then a solution of TsCl (3.17 g, 16 mmol) in THF (10 mL) was added dropwise in 10 min. The reaction was allowed to warm to room temperature and monitored by TLC. The reaction was complete in 4 h. The solvents were removed in vacuo and the residue was dissolved in water (25 mL) and extracted with ethyl acetate (3 × 30 mL). The organic phases were combined, dried over Na_2SO_4 and then concentrated in vacuo. The residue was purified by chromatography (eluting with ethyl acetate/petroleum ether, *v*/*v* = 1:5) to give **8c** (1.79 g, 92%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, *J* = 7.7 Hz, 2H), 7.65 (d, *J* = 8.2 Hz, 4H), 7.44 (t, *J* = 7.3 Hz, 2H), 7.31 (d, *J* = 8.2 Hz, 2H), 7.23 (m, 6H), 4.20 (t, *J* = 7.0 Hz, 2H), 3.86 (qd, *J* = 10.0, 5.4 Hz, 4H), 2.38 (s, 6H), 1.98–1.87 (m, 1H), 1.80–1.67 (m, 2H), 1.42–1.30 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 145.08, 140.24, 132.40, 129.97, 127.82, 125.79, 122.90, 120.39, 119.02, 108.51, 68.43, 42.62, 37.90, 25.83, 24.71, 21.62; IR (KBr): ν 725 (m), 751 (s), 792 (w), 1126 (w), 1201 (m), 1326 (m), 1371 (w), 1459 (m), 1471 (m), 1599 (w), 1626 (w), 2853 (w), 2954 (w), 3048 (vw) cm^{-1}; MS (ESI+): *m/z* = 592.0 $[M+H]^+$.

*9-[5-Azido-4-(azidomethyl)pentyl]-9H-carbazole (**8d**)*

Compound **8c** (0.98 g, 1.7 mmol) was dissolved in DMF (15 mL) and sodium azide (0.86 g, 13 mmol) was added. The suspension was stirred at 60 °C overnight. After being cooled to room temperature, the reaction mixture was treated with brine (50 mL) and the solution was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were washed with brine (3 × 50 mL), dried over Na_2SO_4 and concentrated in vacuo to give the crude product, which was further purified by chromatography (eluting with ethyl acetate/petroleum ether, *v*/*v* = 1:15) to give **8d** (0.31 g, 57%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, *J* = 7.7 Hz, 2H), 7.46 (ddd, *J* = 8.2, 7.3, 1.1 Hz, 2H), 7.36 (d, *J* = 8.2 Hz, 2H), 7.28–7.18 (m, 2H), 4.28 (t, *J* = 7.1 Hz, 2H), 3.34–3.17 (m, 4H), 1.96–1.77 (m, 2H), 1.76–1.63 (m, 1H), 1.46–1.36 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.28, 125.76, 122.94, 120.46, 119.01, 108.46, 52.41, 42.86, 38.54, 27.09, 26.12; IR (KBr): ν 726 (m), 750 (m), 782 (w), 1127 (w), 1159 (m), 1202 (m), 1279 (w), 1331 (m), 1366 (w), 1422 (m), 1442 (m), 1486 (m), 1593 (w), 1627 (w), 2108 (s), 2851 (w), 2951 (w), 3052 (vw) cm^{-1}; MS (ESI+): *m/z* = 334.1 $[M+H]^+$.

*1,3-Bis-[4-(1,5,9-triazacylododec-1-ylmethyl)-1,2,3-triazol-1-yl]-2-[3-(9H-carbazol-9-yl)propyl]propane (**4b**)*

To a solution of **7** (0.38 g, 0.94 mmol) in H_2O/*tert*-butanol (1/1, 10 mL) were added **8d** (0.16 g, 0.47 mmol), $CuSO_4$ (8 mg, 0.05 mmol), and sodium ascorbate (28 mg, 0.1 mmol) under argon. The solution was stirred at room temperature for 24 h. Then the mixture was poured into water and filtered. The solid was washed with water and dried in vacuo, after which the solid was added into HCl solution (Prepared by slowly adding 0.5 mL of acetyl chloride into 8 mL of methanol and then stirring for 5 min at 0 °C) and stirred for 1 h. Then the solvents were removed in vacuo and the resulting solid was dried to give **4b** as its hydrochloric salt (0.37 g, 81%). ^{1}H NMR (400 MHz, D_2O) δ 8.01 (d, *J* = 7.7 Hz, 2H), 7.56 (s, 2H), 7.39 (t, *J* = 7.6 Hz, 2H), 7.29 (d, *J* = 8.3 Hz, 2H), 7.17 (t, *J* = 7.4 Hz, 2H), 4.10 (m, 6H), 3.92 (s, 4H), 3.44–3.11 (m, 24H), 2.38–2.30 (m, 1H), 2.20 (m, 6H), 2.00 (s, 8H), 1.63 (s, 2H); ^{13}C NMR (101 MHz, D_2O) δ 140.13, 137.45, 127.18, 126.22, 121.98, 120.38, 119.23, 109.42, 51.11, 48.56, 47.46, 42.87, 41.64, 40.73, 38.90, 26.00, 24.93, 20.00, 18.87; IR (KBr): ν 727 (w), 757 (w), 858 (w), 996 (w), 1056 (w), 1149 (w), 1233 (w), 1326 (w), 1458 (m), 1483 (m), 1590 (w), 2752 (m), 2951 (m), 3422 cm^{-1} (s); HRMS (ES+) calcd for $C_{42}H_{66}N_{13}$ $(M+H)^+$: 752.5564, found 752.5551.

2.3 Agarose gel electrophoresis

The condensing agents in Tris buffer (50 mM, pH 7.4) were added to the negatively supercoiled pUC18 DNA (9 ng/μL) with a total volume of 20 μL, the mixture was incubated for

5 min at room temperature. Then 2 μL of loading buffer (0.03% bromophenol blue, 0.03% xylene cyanol, 60 mM EDTA, 60% glycerol, 10 mM Tris-HCl, pH 7.6) was added. The resulted solutions were then analyzed by electrophoresis for 5 min at 80 V and 30 min at 100 V on a 0.7% agarose gel in 1 × TAE buffer. The gel was stained with Goldview II and photographed on an UVP EC3 visible imaging system.

2.4 Atomic force microscopy

AFM images were obtained in the air at room temperature with a Veeco atomic force microscope. Scans were run at a rate of 1–3 Hz operating in tapping mode using conical-shaped Si tips integrated to nano-crystalline Si cantilevers with an average resonance frequency of 280 kHz. The images were analyzed with the software accompanying with the imaging module. First freshly cleaved mica was used as substrate for all AFM imaging. Pretreatment of mica was necessary to promote electrostatic immobilization between the condensates and mica. Thus 15 μL of a 10 mM $NiCl_2$ solution was deposited onto the surface of mica for 10 min. The mica was then thoroughly rinsed with pure water to prevent the formation of salt crystals on the surface. Totally 8 μL of DNA solution was spotted onto the pretreated mica and incubated for 5 min, after which the mica was thoroughly rinsed with water, dried under a gentle steam of argon and analyzed by AFM in air condition. The DNA solutions were prepared according to the following procedure. A solution of pUC18 DNA at 9 μg/mL (Tris buffer 5 mM, pH 7.4, $MgCl_2$ 1 mM) was taken in the absence and presence of condensing agents, in which $MgCl_2$ was used optimally in order to facilitate the adhesion of DNA onto mica for better viewing.

2.5 Dynamic light scattering

The hydrodynamic diameter distribution of DNA nanoparticles condensed by different agents was measured by dynamic laser light scattering (DLS) at 25 °C. The scattering angle was set to 90°. DNA solutions (4 μg/mL) were prepared in the presence of condensing agents (Tris buffer 50 mM, pH 7.4) with deionised Milli-Q water (18.25 MΩ). The mixture was allowed to stand for 5 min at room temperature and then 40 μL of the solution was transferred into the standard quartz cuvette for measurement. Typically, 10 runs were measured for each solution, with the average of all the runs reported.

2.6 EB displacement assay

The EB displacement assays were measured in Tris buffer (50 mM, pH 7.4) at room temperature on A Cary Eclipse Luminescence Spectrometer. Along the addition of the solution of condensing agent into the EB-DNA ([EB] = 4 μM, [CT-DNA] = 80 μM) system, the fluorescence spectra of the mixture was recorded after incubation for 2 min (excitement wavelength 524 nm). The apparent binding constant (K_{app}) was calculated using the equation: K_{EB}·[EB] = K_{app}·[condensing agent], where $K_{EB} = 1.0 \times 10^6$ M^{-1}, [EB] = 4 μM, and [condensing agent] was the concentration at which a 50% reduction of the fluorescence had occurred.

2.7 Viscosity titration assay

A Ubblehode viscometer was used to measure the viscosity at 37.0 ± 0.1 °C. CT-DNA (500 μM) was applied in the experiments. Specific viscosity values were calculated from the observed flow time of DNA solutions (t) in the presence of condensing agents corrected for the buffer alone (t_0), $\eta = (t - t_0)/t_0$. The data were presented as $(\eta/\eta_0)^{1/3}$ vs. [condensing agent]/[DNA], where η is the specific viscosity of DNA in the presence of the condesing agents and η_0 is the specific viscosity of DNA alone in Tris-HCl buffer (10 mM, pH 7.4).

2.8 Ionic strength effect

Gel electrophoresis was applied to evaluate the influence of ionic strength on DNA condensation at room temperature. pUC18 DNA (9 μg/mL) was treated with the condensing agents in Tris buffer (50 mM, pH 7.4) and incubated for 5 min in the presence of a range of NaCl concentrations.

2.9 Release of compact DNA

The reversibility of DNA condensation induced by the condensing agents was investigated by agarose gel electrophoresis. pUC18 DNA (9 μg/mL) was treated with the condensing agents in Tris buffer (50 mM, pH 7.4) at room temperature. After the incubation for 5 min to facilitate the condensation, the solutions were treated with NaCl (300 mM) and analyzed by electrophoresis according to the method mentioned previously.

2.10 Cytotoxicity assay

The cytotoxicity of condensing agents toward HepG2 and T98G cell lines were tested by MTT assay (MTT = 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). T98G cells were cultured in minimum essential medium (MEM, Gibco) supplemented with fetal bovine serum (10%, *v*/*v*) in a humid atmosphere containing 5% CO_2 at 37 °C. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (15%, *v*/*v*). After 48 h of incubation in the medium, the cells were seeded in 96-well plates at 5×10^5 cells per well and cultured for another 24 h. Then the cells were treated with different concentrations of condensing agents for 24 h, after which the medium was removed and 10 μL of

MTT (5 mg/mL) was added to wells along with 90 μL of culture medium. The cells were incubated for 4 h and the MTT containing medium was replaced with 110 μL of DMSO. Finally the plates were oscillated for 10 min to fully dissolve the formazan crystal formed by living cells in the wells. The absorbance of the purple formazan was recorded at 490 nm using a Bio-Rad 680 plate reader. The relative viability of the cells was calculated based on the data of four parallel tests by comparing to the controls.

3 Results and discussion

3.1 Syntheses of bifunctional molecules

The syntheses of target molecules were illustrated in Figure 2. Four triazole compounds **1**, **2** and **4**(**a**, **b**) were achieved by copper mediated click reactions between compound **7** and various azides, followed by the removal of the *N*-Boc protecting group using HCl methanol solution. The azides were prepared from the corresponding bromides or tosylate. Among them, ethyl azide was generated *in situ* for the click reaction. The azide **8d** was synthesized in a four-step sequence: alkylation reaction between **5b** and diethyl malonate, reduction of the ester, followed by tosylation and displacement with sodium azide. Compound **3** was obtained through the alkylation reaction between **5b** and **9** in high yield according to the literature procedure [25]. All the target molecules were characterized by ^{1}H NMR, ^{13}C NMR, HRMS and IR.

3.2 Gel electrophoresis assay

The DNA condensations induced by the bifunctional molecules were tested by agarose gel electrophoresis assay. Retardation of the DNA bands in the gel is an indication of a decrease in the negative charge on the plasmid DNA and the formation of large sized DNA particles [2, 16, 32]. As shown in Figure 3, DNA retardation was not observed when the concentration of **1** increased from 40 to 300 μM. In contrast, the amount of supercoiled closed circular pUC18 DNA (Form I) diminished gradually and the condensed DNA appeared in the gel loading wells when the concentration of **2a** and **2b** increased from 60 to 300 μM. For compounds **3** and **4b**, the strongly condensed DNA remained in the gel loading wells when the concentration reached 120 μM for **3** and 80 μM for **4b**, no form I of pUC18 DNA was detected. Compared to **2a** and **2b**, **4b** containing two [12]aneN$_3$ units induced DNA much more effectively. In a controlled experiment, **4a** which contains two [12]aneN$_3$ units but no carbazole unit failed to induce DNA condensation at the concentration of 300 μM.

The above results demonstrate that the presence of both macrocyclic polyamine and carbazol groups are essential for the bifunctional molecules to show effective DNA condensing ability. In addition, the linkage between the two functional units also affects the DNA condensing activity (e.g. compound **3** without triazole moiety in the linkage is much more effective than **2b** in condensing DNA).

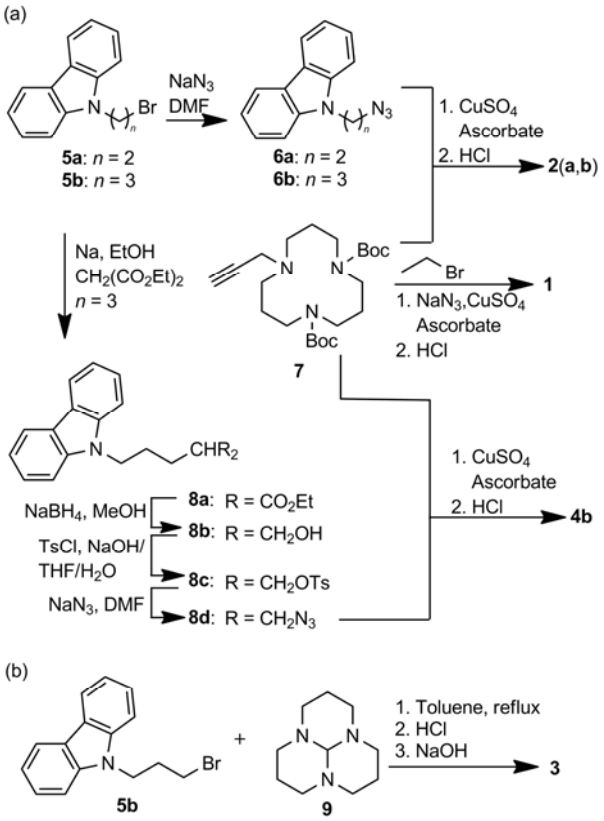

Figure 2 Synthetic routes to the bifunctional molecules.

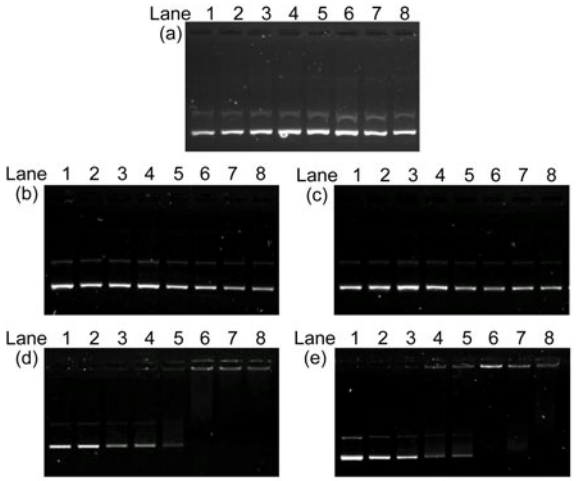

Figure 3 Agarose gel electrophoresis assay to investigate the pUC18 DNA condensation induced by different concentrations of **1** (a), **2a** (b), **2b** (c), **3** (d) and **4b** (e) in Tris-HCl buffer (50 mM, pH 7.4). The DNA concentration is 9 μg/mL. Lane 1, DNA control; Lanes 2–8: 40, 60, 80, 100, 120, 200, 300 μM for DNA + **1** (a) and DNA + **3** (d); Lanes 2–8, 60, 80, 100, 120, 160, 200, 300 μM for DNA + **2a** (b) and DNA + **2b** (c)**;** Lanes 2–8: 10, 20, 40, 60, 80, 100, 120 μM for DNA + **4b** (e).

Compared to other organic condensing agents such as spermine and speramidine [33], **3** and **4b** can induce DNA condensation more efficiently on the micromolar scale and in the absence of Mg^{2+}. When compared to the results of naphthyl modified [12]aneN_3 derivatives [22], the incorporation of carbazol moiety in the bifunctional macrocyclic polyamines further decreased the minimum concentration needed to induce plasmid DNA condensation. With these encouraging results, the DNA condensation induced by **3** and **4b** was further investigated.

3.3 Atomic force microscopy

To gain detailed structural information of the condensates, atomic force microscopy (AFM) assay was carried out. Figure 4 shows the typical AFM images of pUC18 in the absence and presence of condensing agents. In the absence of **3** and **4b**, the naked DNA existed as relaxed circles with slight twisting of the strands (Figure 4(a)), which is characteristic of uncondensed DNA. Upon the addition of 60 μM of **3** (Figure 4(b)) or **4b** (Figure 4(d)), particles of condensed DNA started to appear, coexisting with free circular DNA. As the concentration of **3** (Figure 4(c)) and **4b** (Figure 4(e)) increased to 120 μM, the DNA was totally condensed into particles. The average diameter of the condensed DNA particles was about 100 nm. The AFM images clearly demonstrate the effective DNA condensation ability of **3** and **4b**.

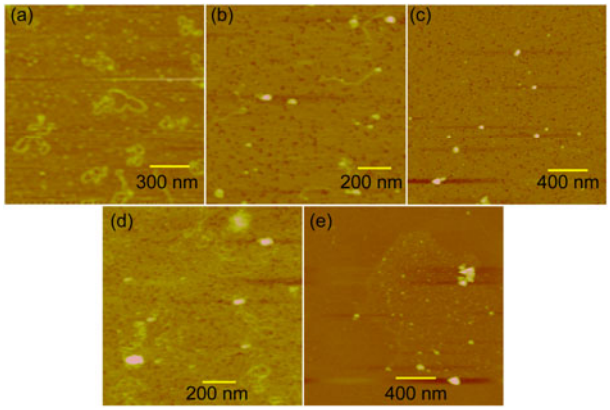

Figure 4 AFM images of pUC18 DNA (9 μg/mL) and its condensation induced by bifunctional molecules in Tris-HCl buffer (5 mM, pH 7.4): (a) DNA; (b) DNA + 60 μM **3**; (c) DNA + 120 μM **3**; (d) DNA + 60 μM **4b**; (e) DNA + 120 μM **4b**.

3.4 Dynamic light scattering assay

The DNA condensates induced by **3** and **4b** in solution were further investigated using dynamic light scattering (DLS). The distribution of hydrodynamic diameters of the DNA particles was measured at a scattering angle of 90°. As shown in Figure 5, the effective hydrodynamic diameters of the DNA particles condensed by 60 and 120 μM of **3** and **4b** were about 213, 517, 245, and 330 nm, respectively. Consistent with the examples reported in literatures [34, 35], the sizes of the DNA particles increased with the concentrations of condensing agents. The DLS results provided further evidence supporting DNA condensation in solution. The different size of the condensed DNA particles in AFM and DLS experiments can be attributed to the different sampling techniques. For AFM, the samples were dried, causing dehydration of the complex and resulting in the decrease in the apparent size of the condensed particle. DLS provides *z* average values in solution in which larger particles contribute more than smaller ones [35].

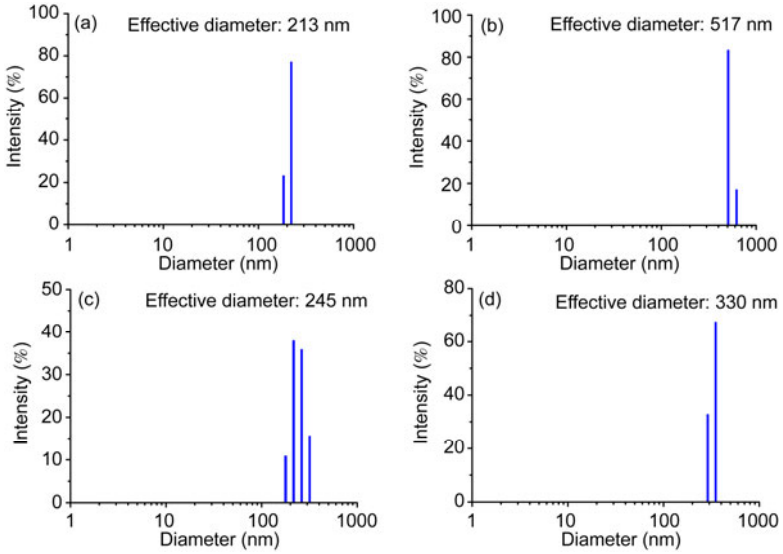

Figure 5 Hydrodynamic diameter distributions of pUC18 DNA particles condensed by (a) 60 μM **3**, (b) 120 μM **3**, (c) 60 μM **4b** and (d) 120 μM **4b** in Tris-HCl buffer (50 mM, pH 7.4) at a scattering angle of 90° at 25 °C. The DNA concentration is 4 μg/mL.

3.5 EB displacement assay

It is well known that the emission intensity of ethidium bromide (EB) can be greatly enhanced upon intercalating with DNA. However, the emission intensity of DNA bound EB can be quenched in the presence of other DNA interclators due to the displacement of EB from DNA [16, 17]. Thus the DNA binding affinity of **3** and **4b** were investigated using the EB displacement assay. The apparent binding constants (K_{app}) of the condensing agents were determined by the EB displacement assay. As shown in Figure 6, the fluorescence intensity of the EB-bound CT-DNA decreased dramatically after the addition of condensing agents, suggesting that **3** and **4b** can effectively replace the DNA bound EB. According to the data obtained from EB displacement assay, K_{app} of **3** and **4b** were calculated to be 2.5 × 10^5 M^{-1} and 5.0 × 10^5 M^{-1}, respectively. The results demonstrate that **3** and **4b** can strongly bind with DNA, and **4b** is better than **3** in DNA binding, which is consistent with the result from gel electrophoresis assay.

3.6 Viscosity titration assay

In order to elucidate the different roles played by the two functional units in compounds **3** and **4b** for the DNA condensation, several additional experiments were carried out. DNA viscosity titration assay is used to determine the binding mode of small molecule with DNA. It is well known that only the intercalation interaction among the three non-covalent binding modes can increase the viscosity of DNA significantly [36, 37]. In contrast, groove binding and electrostatic binding do not show pronounced effect on the viscosity. As presented in Figure 7, upon the addition of **3/4b**, the viscosity of DNA increased remarkably, which is consistent with the observation from the known intercalator EB. Compared to those of naphthyl modified [12]aneN_3 derivatives in our previous work [22], the above results clearly demonstrate that carbazolyl derived **3** and **4b** can bind with DNA through intercalation mode.

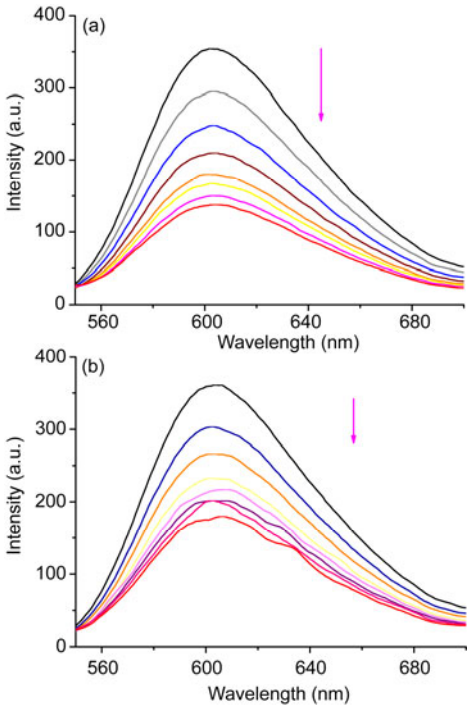

Figure 6 Fluorescence quenching curves of EB bound CT-DNA by **3** (a) and **4b** (b) in Tris-HCl buffer (50 mM, pH 7.4) (λ_{ex} = 524 nm, [EB] = 4 μM, [DNA] = 80 μM, 25 °C). The arrows show the intensity changes on increasing the concentration of the condensing agents from 0 to 150 μM.

3.7 Ionic strength effect

To further understand the binding nature of the condensing agents with DNA, the effect of ionic strength on DNA condensation was also investigated by agarose gel electrophoresis assay. As shown in Figure 8, the amount of non-condensed DNA increased obviously as the NaCl concentration gradually went up from 0 to 400 mM, suggesting that the condensing ability of **3** and **4b** was weakened by the increasing ionic strength. This is an indication of electrostatic interactions between **3/4b** and DNA. Excess salt can partially neutralize the phosphate backbone, which in turn decreases the affinity of condensing agents with DNA [38]. Fortunately, DNA condensation was still detectable as the concentration of NaCl raised above that of physiological value. Such inhibiting effects of NaCl clearly indicate that electrostatic interaction resulted from [12]aneN_3 functional units plays an important role in driving the condensation process. Together with the results of DNA viscosity titration assay and structure activity relationship, we conclude that **3/4b** bind with DNA through both electrostatic interaction and intercalation binding modes.

3.8 Release of compact DNA

The release of DNA from its compact state is a prerequisite

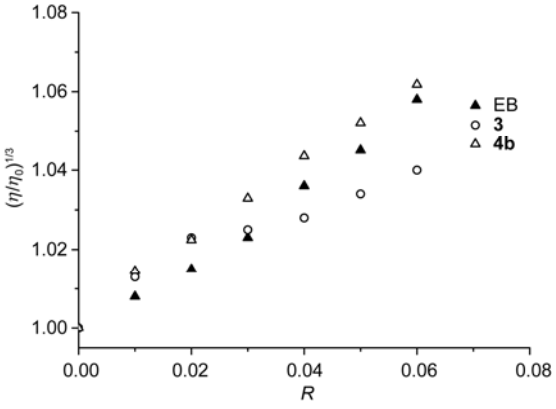

Figure 7 Relative specific viscosities of CT-DNA at 37 °C in 10 mM Tris-HCl buffer (pH 7.4) as the function of the concentration ratio (*R*) of condensing agents and CT-DNA (500 μM).

for efficient gene expression. Thus an ideal DNA condensing agent should not only have the ability to condense DNA, but also release DNA from the compact state under proper conditions. General procedures used to release the compact DNA include addition of additives [34, 39], linkage breaking [40, 41], pH jumping [42] and so on [39]. Herein the compact DNA was treated with concentrated NaCl solution to trigger its dissociation from the condensing agents. As shown in Figure 9 of gel electrophoresis assay, the condensed DNA was partly released after being treated with 300 mM NaCl, suggesting the reversibility of DNA condensation induced by **3**/**4b**. The release of compact DNA may be attributed to the electrostatic competition between the condensing agents and Na^+ with negatively charged phosphate backbone of DNA. As a result, the interaction between DNA and the condensing agents was weakened, and the repulsion between DNA segments was enhanced, leading to the release of DNA. No cell can survive at such a high concentration of NaCl, but the above experiment showed that the DNA condensation induced by **3** and **4b** is potentially reversible.

As a short summary, the effective condensation ability of **3** and **4b** mainly came from two aspects: (i) the electrostatic effect from the protonated [12]aneN_3 units whose positive charges could neutralize the negative charges of DNA and reduce the repulsion between the nucleotides; (ii) the intercalative effect from the carbazole unit which could further enhance the compact of DNA.

3.9 Cytotoxicity

As potential gene vectors, the condensing agents are desired to have low toxic effect on cells. Thus the cytotoxicity of **3** and **4b** was tested by MTT assay against HepG2 and T98G cell lines following the literature methods [22, 43]. As shown in Figure 10, the cytotoxicity of **3** towards HepG2 and T98G rose significantly as the increase of dose. In contrast, **4b** showed very weak toxicity towards HepG2 and T98G. The viabilities of HepG2 and T98G were still above 70% when the concentration of **4b** reached 80 μM, the minimum concentration needed for DNA condensation. The results demonstrate that the toxicity of **3** is too high, but **4b** should be suitable for the development of non-viral gene vector due to its low toxicity.

4 Conclusions

A series of bifunctional macrocyclic polyamines containing carbazol group were designed and synthesized as effective DNA condensation agents. Agrose gel electrophoresis, AFM and DLS assays showed that compound **3** and **4b** could effectively induce plasmid DNA condensation reversibly at low concentrations (120 μM for **3** and 80 μM for **4b**). The larger planar aromatic ring size of carbazol moiety, when compared to that of naphthyl group, improved the condensation ability of the bifunctional molecules. The fluorescence spectra, ionic strength effects and DNA viscosity assay demonstrated that **3** and **4b** could interact strongly with DNA through electrostatic and intercalation binding

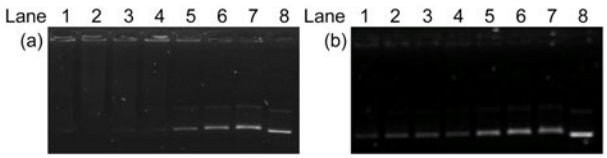

Figure 8 Agarose gel electrophoresis assay to investigate the effect of ionic strength on pUC18 DNA condensation induced by **3** (a) and **4b** (b) in Tris-HCl buffer (50 mM, pH 7.4): Lanes 1–7, [NaCl] = 0, 10, 50, 100, 200, 300, 400 mM; Lane 8, DNA control. The concentration of DNA, **3** and **4b** are 9 μg/mL, 120 μM and 80 μM, respectively.

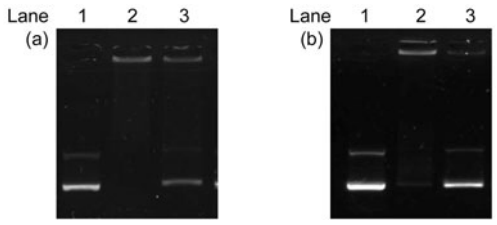

Figure 9 Agarose gel electrophoresis assay to investigate the reversibility of DNA condensation induced by **3** (a) and **4b** (b) in Tris-HCl buffer (50 mM, pH 7.4): Lane 1, DNA control; Lane 2, DNA + **3** (a) or **4b** (b); Lane 3: DNA + **3** (a) or **4b** (b), then treated with NaCl. The concentrations of DNA, **3**, **4b** and NaCl are 9 μg/mL, 120 μM, 80 μM and 300 mM, respectively.

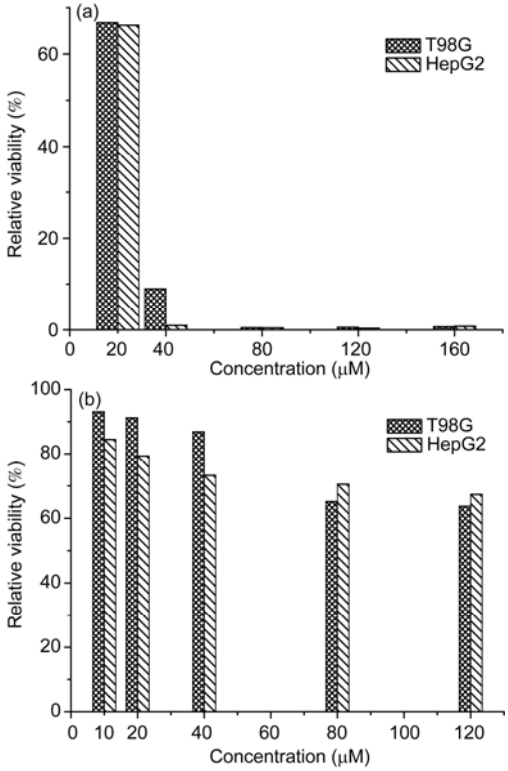

Figure 10 The cytotoxicity of **3** (a) and **4b** (b) toward T98G and HepG2 respectively, after incubation at 37 °C for 24 h.

mode. The structure activity relationship confirmed that both macrocyclic polyamine and carbazol groups are essential for the effective DNA condensing ability. MTT assay showed that the incorporation of triazole units in the bifunctional **4b** greatly lowered its cytotoxicity, indicating its potential as nonviral gene vector. The work further proved that proper combination of the electric-static moieties and the intercalative aromatic units can lead to effective DNA condensation agents with lower cytotoxicity and good water solubility. The gene delivery ability of the bifunctional molecules is being investigated in our lab.

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